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Direct radiation effects on the structure and stability of collagen and other proteins

Mathieu Lalande,[a] Lucas Schwob,[b] Violaine Vizcaino,[a] Fabien Chirot,[c] Philippe Dugourd,[d] Thomas Schlathölter[e] and Jean-Christophe Poully*[a]

Abstract: In this review, we survey recent progress aiming at understanding the direct effects of radiation on the structure and stability of collagen, the most abundant protein in the human body, and other proteins. Special emphasis is put on the triple helical structure of collagen as studied by means of collagen mimetic peptides. The emerging patterns are the dose-dependence of radiation processes and their abundance, the crucial role of radicals in covalent bond formation (cross-linking) or cleavage, and the influence of the radiation energy and nature. Future research should allow answering fundamental questions such as charge transfer and fragmentation dynamics triggered by ionization, but also developing applications like protein-based biomaterials, notably with properties controlled by irradiation.

Proteins are crucial for cellular functions of living organisms, where proteins for instance catalyze chemical reactions, perform signal transduction and transport ligands, allow motility, and replicate DNA. Moreover, mechanical properties of the extracellular matrix (ECM) such as stiffness and elasticity, and ECM functions (intercellular communication, cell adhesion, tissue protection…) are mediated by proteins. The ECM is mainly composed of glycoproteins, proteoglycans, polysaccharides, elastin and collagen. The latter is the major protein in the ECM, and provides most of its frame and stiffness, *via* collagen supramolecular structures made of assemblies of fibers. Collagen is also the most abundant protein in the human body, present in a wide range of different (mostly connective) tissues, and is the main component of cartilage, tendons, ligaments and skin. Characteristic properties of collagen such as primary to quaternary structures, stability and mechanical properties are tissue-specific and to date 27 unique collagen types have been identified in vertebrates. Common to all these members of the collagen family is the presence of long sequences (from 100 to 1000 residues) made of repeated XYG triplets (G being glycine, X and Y any other amino acids). These sequences form the triple helical secondary structure that is typical of collagen but which can also be found in other proteins such as complement protein C1.[1] The structure-function relationship is particularly important for collagen, and it appears at different scales from molecular to macroscopic. The length of single collagen proteins can vary from 14 to 2400 nm,[2] fibril lengths are on the µm scale and fibers can extend to the mm range.[3] This fibrous structure is even visible in tissues like tendons, which drove very early interest for deciphering the molecular basis of their mechanical properties. Indeed, in 1938 the first structural study on collagen reports on X-ray diffraction of a rat tendon.[4] Later, a structure with better resolution was reported and is shown in Figure 1. As can be seen, the triple helix is composed of three protein strands tightly wound around each other, with all peptide bonds in *trans* configuration, allowing for a strong interstrand H-bonding network between backbone amide groups commonly referred to as Rich and Crick II.[5] Each of the strands has a polyproline II secondary structure, which is consistent with the very high (around 20%) proline content in collagen triple helical domains. An unusually high fraction of these proline residues located at the Y position are post-translationally modified by substituting one side-chain H atom by one OH group. The resulting hydroxyprolines (denoted O in the amino-acid letter code) are known to stabilize the triple helix.[6] Mandatory for the existence of the triple helical structure are a 1-residue staggering of the three strands as well as the presence of glycine every three residues, because it has the smallest side-chain of all amino acids and therefore sterically allows the three backbones to be closely bound. This requirement is so important that mutations of this glycine (called collagen interruptions) can lead to destabilization of the helix, collagen misfolding and the emergence of diseases such as *osteogenesis imperfecta*.[7]

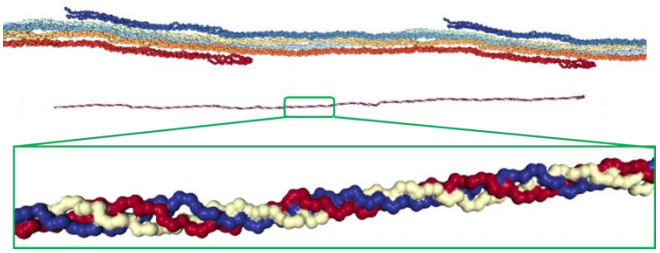

Figure 1 crystal structure of collagen from an X-ray diffraction experiment on a tendon of *Rattus Norvegicus* (PDB 3HQV).[8] On the top, each triple helix in the fibril is depicted in rainbow colors, from one end to another. In the middle, a single triple helix is shown, with a zoom at the bottom where each protein is colored differently.

Mutations in proteins can be induced by external factors, therefore probing the response of biological matter under their influence is crucial for understanding the way living organisms survive and adapt to their environment. In particular, biological systems have always been interacting with radiation, especially light from the sun. Advanced medical techniques use non-ionizing but also ionizing radiation such as X-rays in radiography, computed tomography (CT), mammography, angiography and fluoroscopy. Higher energy (MeV) X-rays are also employed for the treatment of certain types of cancer by radiotherapy, as

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these photons are able to penetrate the body and kill tumor cells. Ion beams (mainly protons but also carbon ions) at MeV energies are also used in hadrontherapy. This technique has been receiving a growing interest over the last decades because of its advantages as compared to radiotherapy, in terms of precise targeting of the tumor, superior ballistic properties and biological efficiency to kill cells.[9] Another way of targeting cancer cells is to attach radioisotopes to ligands that specifically bind to membrane receptors: this is called radioligand therapy. Radiopharmaceuticals can also be used for molecular imaging, in positron emission tomography or single photo emission computed tomography. UV light, X and gamma rays as well as electron beams are also routinely used to sterilize food or medical products.[10]

To understand the effects of ionizing radiation on proteins, it is crucial to control the experimental conditions, especially in terms of temperature and phase. Irradiation of a solution at room temperature leads to the formation of free radicals from the solvent (*i.e.* hydroxyl from water for instance) as main primary products. These species then chemically react with proteins, leading to secondary processes such as backbone cleavage and in some cases aggregation of the fragments formed,[11] cross-linking between proteins forming larger systems or even nanoparticles with diameter in the 10 nm range,[12] and generally quenching but also enhancement of biological activity.[13] The effect on activity is thought to be due to a radio-induced conformational change, in the same line as other reports that give evidence for unfolding of proteins after irradiation.[14,15] All these indirect effects require diffusion of free radicals from the solvent to the protein, which occurs at rates that decrease by several orders of magnitude from room to cryogenic temperatures.[16] This free radical-mediated mechanism is the reason why the radio-induced loss of protein activity with rising temperature does not depend on the protein in solution. Therefore, freezing proteins allows studying the direct effects of radiation. Studies aiming at understanding these effects have been reviewed more than 10 years ago.[16,17] At that time, no gas-phase study on proteins had been performed, thus lyophilized, crystallized and dried or frozen samples were irradiated and direct effects analyzed thanks to experimental techniques such as radiation target analysis and capillary electrophoresis, IR, UV and Electron Paramagnetic Resonance (EPR) spectroscopies or gas chromatography. For instance, the two latter have been employed to investigate homopolymers of amino acids, and it was established that loss of side chains occurs regardless of the amino acid, whereas backbone cleavage is quenched in the case of tyrosine and phenylalanine, presumably due to radical trapping at their aromatic rings.[18] In proteins, cleavages also occur, and their locations on the backbone have been found to be random for some of them, but specific for others, mostly non-membrane proteins. For instance, fragmentation is more likely at loops and turns between α-helices and β-sheets of aspartate transcarbamylase.[19] Interestingly, covalent bonds cleavage does not necessarily induce loss of the protein secondary, tertiary or quaternary structure.[16] However, in some cases, irradiation leads to separation of noncovalently-bound protein subunits.[20] This has been attributed to energy transfer between sub-units after irradiation, like in β-galactosidase, which is active as a tetramer but inactivated after fragmentation in monomers.[21]

Although some of the aspects of the structure and stability of collagen as well as direct effects of radiation on proteins had already been described 10 years ago, other points remained poorly understood, as for instance E. S. Kempner[16] wrote, “the nature of molecular damage as a function of energy deposited in the primary ionization deserve[d] further investigation”. In this review, we survey the efforts undertaken during the last decade towards a description and understanding of direct effects on the primary, secondary and tertiary structure as well as stability of proteins, especially collagen, upon irradiation by photons, ions and electrons.

1. Structure and stability of collagen mimetic peptide assemblies

A large amount of information on the structural properties and stability of collagen has been gained by studying systems smaller than entire collagen proteins or even collagen fragments. Collagen mimetic peptides (CMP) are for instance easier to crystallize for X-ray diffraction studies. As a consequence, better resolution and thus more precise structural details can be achieved. CMP can be synthesized to include any mutation in a desired amino acid sequence. It was early demonstrated that the $(PPG)_{10}$ sequence forms stable triple helices in solution[22] and in a crystal.[23] The latter study clearly revealed the H-bonds linking a given peptide backbone to the two others (see Figure 2).

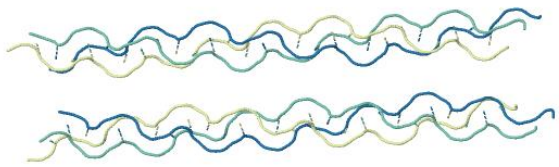

Figure 2 crystal structure of the two triple helices of the $(PPG)_{10}$ collagen-mimetic peptide (PDB 1K6F).[24] Each peptide of a given triple helix is depicted in a different color, and intermolecular H-bonds are drawn in dashes.

The melting temperature T_m and increase of enthalpy ΔH^0 for denaturation of $(PPG)_{10}$ have been reported to be $T_m = 24.5°C$ and $\Delta H^0 = 150 - 180\ \mathrm{kJ.mol^{-1}}$,[25] respectively. Hydroxylation of all prolines in Y position leads to the $(POG)_{10}$ peptide, which forms an even more stable triple helix with $T_m = 56.9°C$ and $\Delta H^0 = 370 - 390\ \mathrm{kJ.mol^{-1}}$. This is consistent with the stabilization observed for triple helical domains of collagen proteins, even if their hydroxyproline content is lower than that of $(POG)_{10}$. Contradictory claims have been reported about the role of hydroxyproline in the X position.[26,27] The mechanism of triple helix stabilization by hydroxyproline is not based on direct intra-helix H-bonds involving the OH group of its side chain, since crystal structures show no such H-bonds. Instead, a water-mediated hydroxyproline-backbone H-bond network is observed, similar to the case of hydration networks in collagen fibrils.[28] However, a series of further studies (see the reviews by Bella[29] and Raines[6] for details) brought evidence that intra-peptidic stereoelectronic effects involving the hydroxyproline OH are mainly responsible for triple helix stabilization.[30,31] A totally different interaction is responsible for stabilization via cystine knots: the intermolecular covalent bond between two sulfur atoms of cysteine residues.[32] Another crucial stabilizing interaction in collagen was discovered thanks to CMP: interstrand salt-bridges between lysine and aspartic or glutamic acid residues.[33] Their strength is due to Coulomb attraction between deprotonated acids and protonated lysine side-chains. The requirement for this interaction to exist is the presence of KGE or KGD sequences.[34] Even though local effects have been successfully applied to predict the stability of synthetic CMP,[33]

extending to collagen proteins is more delicate. Indeed, suppression of a single salt bridge can have long-range impacts, such as micro-unfolding of a proline-poor region of the triple helix, as demonstrated by Xu *et al*.[35] Experiments on CMP have shown that these regions lacking hydroxyproline or proline can form a stable triple helix if they contain another modified residue: O-glycosylated threonine.[27] This modification is covalent binding of a polysaccharide group to the side-chain oxygen of a threonine residue. It explains the stability of some cuticle collagens.[36] Recently, it has been proposed that intermolecular H-bonds between glycosylated hydroxylysine residues stabilize triple helical domains of adiponectin (see Figure 3).[37] Interestingly, Huang *et al*.[38] showed that glycosylated hydroxyproline slightly destabilizes triple helical CMP, but increases their assembly rate, which might be due to these intermolecular interactions. Therefore, the way glycosylation influences the triple helix seems to be different from that of hydroxyproline. We recently shed light on the role of the latter, by performing experiments on the structure and stability of CMP in the gas phase.

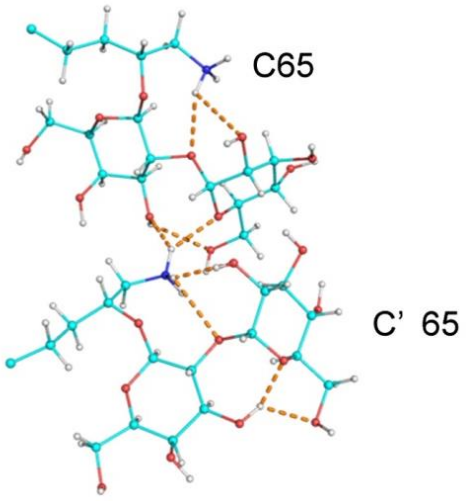

Figure 3 Proposed structure of a region of a peptide trimer mimicking the collagenous domain of adiponectin, showing H-bonds (in dashes) between glycosylated hydroxylysine residues C65 and C'65. Carbons, nitrogens, oxygens and hydrogens are depicted in cyan, blue, red and white, respectively. Adapted from ref.[37] with permission.

The intrinsic structural properties of collagen-related systems were only scarcely investigated: IR[39] and rotational[40] spectroscopic experimental studies focusing on stereoisomers of hydroxyproline found that stereoelectronic effects stabilize the collagen-relevant isomer. Collision-induced dissociation coupled to mass spectrometry has been used to determine the glycosylation site of collagen-related glycopeptides.[41] Assemblies of whole type I, III and V collagen proteins have been put in the gas phase by matrix-assisted laser desorption ionization and detected by mass spectrometry.[42] The polyproline II structure of small collagen-related isolated peptides was investigated by Density-functional theory (DFT)[43], but before our recent work,[44] there was no report on triple-helix models in the gas phase. Therefore, we recently applied tandem ion mobility coupled to mass spectrometry to unravel the intrinsic structure and stability of these systems. Mass spectrometry allows to perfectly control the stoichiometry of the molecular assembly. Ion mobility spectrometry consists in measuring the arrival time of molecular ions after a drift in a tube filled with rare gas: at a given charge state, the more extended the conformation, the higher the number of collisions with gas and thus the arrival time. CMP assemblies were put in the gas phase thanks to electrospray ionization, a technique that does not damage thermally-fragile molecular systems. First, we have shown that protonated $(PPG)_{10}$ and $(POG)_{10}$ peptide trimers are triple helical in the gas phase, if they contain more than seven protons. Thus, water is not required for the triple helix to exist. Furthermore, activation of these systems by low-energy collisions with helium demonstrated that hydroxyproline increases the dissociation energy of the triple helix, which means that intrinsic effects play a crucial role (see Figure 4). Second, the $(PPG)_{10}$ dimer with nine protons keeps its crystal structure without water, consisting in two antiparallel triple helices (see Figure 2), presumably bound by the interaction between their strong dipole moments. This is challenging the claim of non-existence of these small assemblies of triple helices in solution without hydroxyproline.[45] We are currently investigating these systems more deeply, to get insight into the first steps of collagen growth into fibrils. We also plan to study how a controlled number of water molecules change the structure and stability of CMP assemblies.

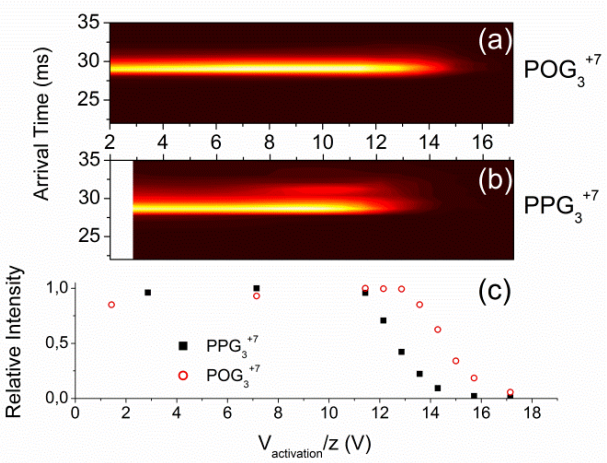

Figure 4 (a) and (b) 2D maps of the arrival time distributions of $[((POG)_{10})_3+7H]^{7+}$ and $[((PPG)_{10})_3+7H]^{7+}$ (noted POG_3^{+7} and PPG_3^{+7}) as a function of collision voltage. (c) Evolution of the relative intensity of POG_3^{+7}, and PPG_3^{+7} as a function of collision voltage. Re-used from ref.[44] with permission.

2. Irradiation by non-ionizing radiation

UV light is a major factor of skin ageing and cancer induction. Since the dermis of skin is mainly composed of a dense extra-cellular matrix containing numerous collagen fibrils, UV irradiation of collagen has been studied by a range of experimental techniques. Particular attention has been given to effects on the characteristic triple-helical 3D structure of collagen, because of its crucial role in the mechanical properties of connective tissues. Investigations on collagen proteins in solution using experimental techniques such as differential scanning calorimetry (DSC) or circular dichroism (CD), but also various chemical analyses, demonstrated that UV irradiation has an effect on their geometrical but also chemical structures. A transition from triple helix to random coil occurs through an intermediate state characterized by Miles *et al*.[46] UV absorption is thought to occur mainly at tyrosine and phenylalanine (tryptophan is almost absent in collagen) aromatic side chains,

creating cross-links between collagen strands but also backbone cleavages.[47,48] These cross-links and cleavages would be due to free radicals created by tyrosine and phenylalanine photoproducts. The intermediate state is more flexible, and thus has a higher entropy than the native triple helix, resulting in a decrease of the entropy change for denaturation, but has a similar enthalpy change.[46] Interestingly, it has been reported that low doses of UV light increase the denaturation temperature of collagen in tendons,[49,50] which would be due to cross-linking between triple helices. At higher doses, atomic force microscopy and DSC showed that the high number of backbone cleavages leads to destruction of collagen fibers, loss of triple helix structure and protein disintegration into small peptides.[51,52] It has been proposed that the radicals created by UV absorption in aromatic residues migrate to proline and then glycine residues, which might finally lead to backbone cleavage.[53] Consistent with this picture, a higher probability of damage for proline-containing CMP as well as preferential glycine-proline backbone cleavage have been observed by mass spectrometry analysis of UV degradation products of different CMP.[54]

Other proteins than collagen have also been studied by means of UV and IR spectroscopy, notably taking advantage of the high flux of synchrotron radiation to reach good signal-over-noise ratios for far UV-CD studies. Heat-induced denaturation of matrix proteins[55] as well as antifreeze protein III[56] has been observed, but this process also occurs upon long enough exposure to UV light. Controlling synchrotron radiation CD UV denaturation can even give information about protein stability and receptor-ligand binding interactions, particularly when thermal studies are inconclusive. For instance, it allowed showing that gold nanoparticles or ligands stabilize human serum albumin upon irradiation in the 185-250 nm wavelength range,[57]

3. Irradiation by ionizing radiation

Proteins are exposed to other radiations than UV light, such as gamma rays and electrons with kinetic energy of about 10 MeV that are routinely used for sterilization of biomaterials. From the studies that have been reported these last two decades, several main conclusions can be drawn: first, the magnitude and nature of direct effects are often highly dose-dependent. Second, radicals created by ionization of atoms or groups within individual proteins are involved in side-chain loss as well as backbone cleavage, but also cross-linking. Third, the nature and energy of the ionizing radiation often play a big role. We will detail all these points for collagen and other proteins in the following section.

Direct effects have usually been reported to start being detectable for doses around 10 kGy, which is higher than indirect effects due to free radicals after irradiation of a solution at room temperature.[58] However, it should be noted that EPR spectroscopy of a powder of bovine hemoglobin (Hb) after irradiation by gamma rays at doses as low as 5 Gy allowed observing a signal assigned to peroxyl and tyrosyl radical formation.[59] The authors attribute this to “the high sensitivity of Hb protein to irradiation”. The signal then rises linearly with increasing dose up to 300 kGy. Interestingly, gamma rays at the same very low dose of 5 Gy have also been found to increase the diameter of collagen fibrils without significant cross-linking.[60] In contrast, Hu *et al.*[61] have studied the modification of collagen thin films by space radiation, which is composed of electromagnetic radiation such as X or gamma rays, but also protons and other ions, and observed extensive cross-linking as well as a decrease in thermal stability of collagen proteins. This thermal behavior might be protein-dependent, since the opposite has been observed for proteins from sunflower meal irradiated by gamma rays between 10 and 50 kGy.[62] It was attributed to cross-linking but might also be due to conformational change. Indeed, a progressive transition from α-helix to β-sheet and random coil secondary structures was observed thanks to FT-IR spectroscopy. This latter process has been reported for different proteins and radiation types, for instance bovine serum albumin (BSA) irradiated by N^+ ions[63] or protons,[64] although the underlying mechanism remains opaque.

In the high dose regime, from 1 to 100 MGy, other processes mainly involving covalent bond cleavage can be observed at the molecular or atomic level by techniques such as Near-Edge X-ray Absorption Fine Structure (NEXAFS) spectroscopy or X-ray diffraction. The latter requires protein crystals and low temperatures, usually below 150 K. In proteins containing cystine, disulfide bond cleavage is usually observed, for instance in acetylcholinesterase and hen white egg lysozyme irradiated by X-rays at 10 MGy.[65] Other bonds are also broken, such as C-H, C-N and C=O from the amide backbone of surface proteins,[66] cytochrome C or BSA.[67] A mechanism common to several polymers containing amide groups has been proposed after C-O cleavage: H transfer from N to the carbonyl C or C_α, forming imine or nitrile groups, respectively (see Figure 5).[67] In the latter case, it leads to fragmentation of the protein backbone.

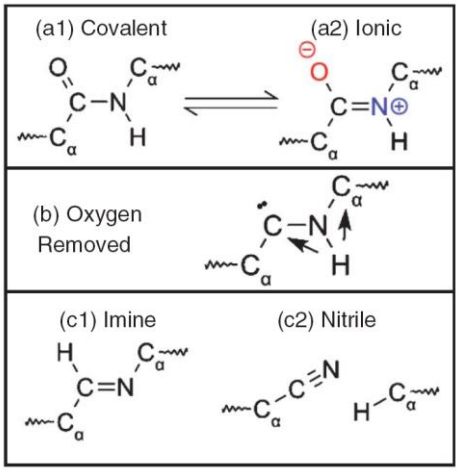

Figure 5 (a) covalent and ionic structures of the amide group ; (b) amide group after O removal and illustration of H transfers ; (c) imine and nitrile groups formed. Re-used from ref.[67] with permission.

C-H and C=O cleavages lead to loss of H and O atoms, which can give rise to H_2 and O_2 when these radicals self-recombine.[68,69] C-O cleavage within serine and threonine side-chains leads to loss of the hydroxyl group.[70] OH loss after C-O cleavage of the tyrosine side-chain is controversial, since it has been reported several times but challenged by recent work.[71] Contradictory results have also been reported about C-C bonds: formation in surface proteins but cleavage in other proteins including hen egg white lysozyme, acetylcholinesterase and chymotrypsin inhibitor.[72] This cleavage explains CO_2 loss from glutamic and aspartic acid side-chains. Interestingly, all these specific atomic displacements have been shown to trigger cooperative movements of protein structural sub-domains.[68] Another characteristic of these cleavages is the involvement of

radicals created by ionization, as we will detail further in the following.

The mechanisms proposed to account for direct effects of radiation on proteins are often radical-mediated. For instance, cross-linking has been attributed to the formation of dityrosine after binding of two tyrosyl radicals (*cf.* Figure 6).[73] Different radicals might play a role in collagen, such as CH_2-$CH_2^{\bullet}$ that has been detected by EPR of collagen samples, irradiated by 2 MeV protons.[74] It is consistent with a recent work by Kornacka *et al.*,[75] who used the same technique and concluded that gamma-rays irradiation at 5 kGy creates radicals at proline side-chains. CO_2 loss from glutamic and aspartic acid side-chains, but also H_2 and O_2 formation as well as backbone cleavage have been suggested to be due to radicals (see previous paragraph). Loss of neutral molecules from side-chains has also been observed from isolated peptides or proteins after ionization. We will see in the next paragraph how the advent of gas-phase studies brought complementary information to condensed-phase investigations, notably by varying the nature and energy of the radiation.

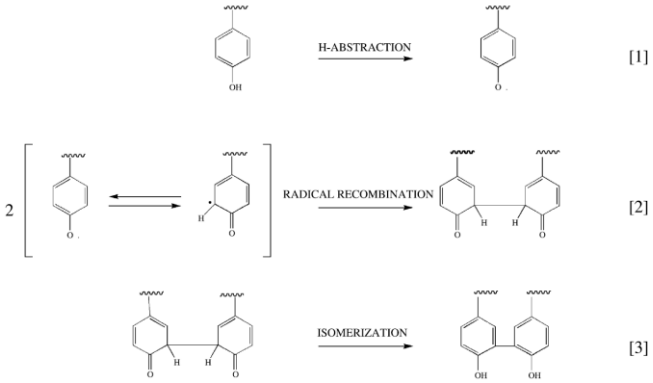

Figure 6 radical-mediated mechanism of dityrosine formation from two tyrosine side-chains. Re-used from ref.[73] with permission.

These last decades, advances in soft sources of gas-phase molecules such as electrospray ionization (ESI) or Matrix-Assisted Laser Desorption Ionization allowed studying larger biologically-relevant systems than DNA bases or amino acids. However, due to the very low current intensity (on the order of 1 pA) delivered by these sources, and even with the highest radiation fluxes available (cyclotrons, lasers, synchrotrons…), ion traps had to be employed in order to irradiate these biomolecules for 0.1-1s to obtain decent signal count rates. In these experiments, the number of interactions can be controlled by measuring the depletion of the precursor ion. Zubarev *et al.*,[76] Giuliani *et al.*[77] as well as Schlathölter *et al.*[78,79] pioneered experiments involving irradiation of mass-over-charge selected peptides and proteins and analysis of the product ions by mass spectrometry. Early studies with electron beams of controlled kinetic energy allowed measuring the first ionization cross-sections and energies of protonated peptides such as substance P or vasopressin.[80] The latter are in the 10-15 eV range, consistent with the ejection of valence electrons. Further work established a 1.1 eV increase of the ionization energy when the protonated peptide charge state increments, which was assigned to Coulomb attraction underwent by the ejected electron.[81] This behavior has been confirmed by photoionization experiments.[82] Much lower photon energy is needed to detach one electron from multiply-deprotonated peptides, which supports the electrostatic picture.[83] After ionization or electron detachment, loss of neutral molecules from the radical species was also detected in the mass spectra, and regarding cations, it was early proposed to come from side-chains as well as the C-terminal carboxylic acid.[84] Later, these losses from ionized peptides were identified as coming from side-chains of specific amino acids such as tyrosine, aspartic and glutamic acids, and serine.[85,86] Photoionization of substance P as a function of photon energy, thanks to synchrotron radiation, allowed measuring appearance energies of a few eV for these losses, consistent with radical-driven processes.[87] We studied a peptidic sequence of type I collagen as well as peptidic models of the collagen triple helix by synchrotron radiation in the VUV and soft X-ray ranges,[88,89] and also observed the loss of neutral molecules with low appearance energy that we attributed to radical-driven processes at amino acid side-chains (see Figure 7), akin to the case of irradiation of proteins in condensed phase (see previous sub-section). However, in contrast, gas-phase radicals were mainly located at aspartic acid and hydroxyproline side-chains. These radicals might be too short-lived to be observed in condensed phase.

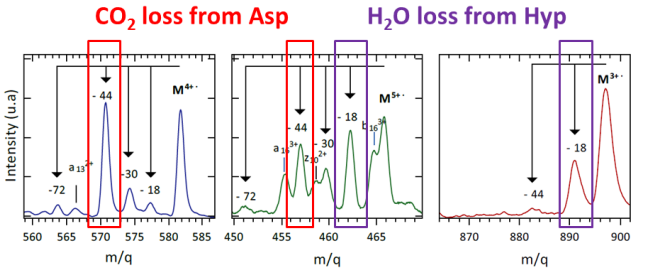

Figure 7 Mass spectra of a protonated type I collagen peptide (left and middle) and of protonated $(POG)_{10}$ (right) after absorption of one 150 eV photon.[88] The mass of neutral molecules lost after ionization is indicated, and losses from aspartic acid as well as hydroxyproline side-chains are highlighted. Reproduced from reference[88] by permission of the PCCP Owner Societies.

At higher photon or electron energy, peptide backbone fragmentation is observed, with an abundance that decreases with peptide size at a given photon energy.[90,91] This has been interpreted as fragmentation in the ground state after redistribution of part of the initial photon energy in the internal degrees of freedom, the latter increasing with peptide size, thus almost quenching fragmentation for proteins.[92] Non-dissociative multiple ionization has even been observed for cytochrome C (see Figure 8), ubiquitin and insulin, after photoabsorption of one soft X-ray photon.[90,93] In this energy range, Auger electron emission occurs and has been shown to induce secondary ionization of melittin.[94] Thanks to NEXAFS spectroscopy and mass spectrometry, gas-phase experiments allowed identifying resonant excitation of 1s electrons of carbon, nitrogen and oxygen atoms to unoccupied molecular orbitals, but also direct ionization for slightly higher photon energies, since Auger decay leads to ejection of one electron for excitation and two electrons for direct ionization (*cf.* Figure 8). It is important to notice that these excitation energies are very similar to those from condensed-phase measurements.[93,95] Non-dissociative ejection of several electrons can also be achieved upon irradiation by ion, as illustrated by experiments with multiply-charged Xe beams on deprotonated and protonated cytochrome C.[96,97] For smaller systems such as protonated peptides, backbone fragmentation as well as side-chain loss of neutral molecules occur, indicating that mechanisms are similar to those regarding electron and photon interactions.[98,99] Interestingly, an ion-specific process has been reported: proton detachment, leading to the formation

of an intact cation without one charge.[100,101] However, it might also come from a reduced species formed by electron capture, as suggested recently.[102] More investigation is needed to clarify this point.

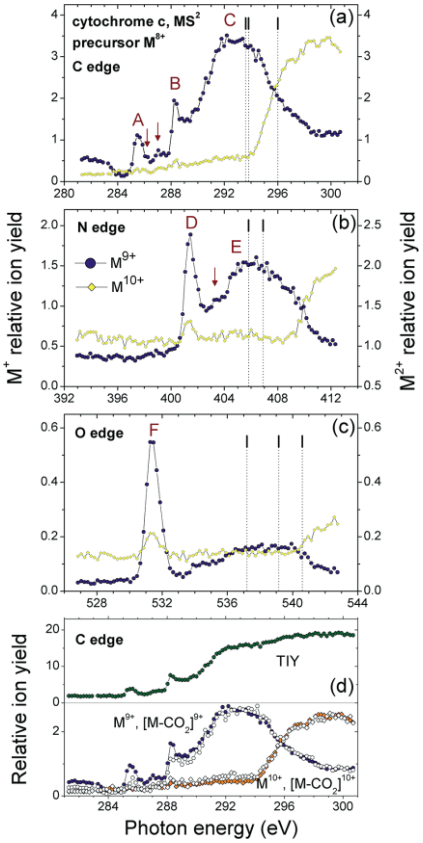

Figure 8 (a), (b), (c): relative yields of non-dissociative single (M^+) and double (M^{2+}) ionization after photoabsorption by [cytochrome C + 8H]$^{8+}$ as a function of photon energy ; (d) total ion yield (top) and CO_2 loss after single and double ionization. A to E indicate resonant transitions of 1s electrons to unoccupied molecular orbitals. Reprinted with permission from reference.[93] Copyright (2012) American Chemical Society.

The role of non-covalent binding on the processes induced by ionizing radiation and the way energy flows through protein subunits started to be studied only recently. To do so, we irradiated $(PPG)_{10}$ and $(POG)_{10}$ peptidic models of the collagen triple helix by means of ionizing photons at the BESSYII synchrotron (Berlin, Germany) and carbon ions at the IRRSUD beamline of the GANIL facility (Caen, France). In the case of VUV photons in the 14-20 eV range, we observed a transition between photoexcitation leading to fragmentation and non-dissociative ionization after single photon absorption (*cf.* Figure 9).[89] Above 20 eV and up to soft X-ray energies (100-600 eV), the internal energy transferred to the system rises with photon energy and leads to more and more fragmentation. When photon energy increases, inter-molecular fragmentation first occurs, thus unfolding the helix and forming isolated peptides, which fragment further if the photon energy is high enough (see Figure 9). Interestingly, this intramolecular fragmentation is dominated by backbone cleavage between glycine and proline residues, as observed in solution after UV irradiation.[54] It supports the role of intrinsic processes not due to the solvent. We also observed an increased stability upon VUV photoabsorption for $(POG)_{10}$ as compared to $(PPG)_{10}$ triple helices, which is consistent with the hydroxyproline effect already observed upon collision with helium gas (see section 1), but also with the well-known effect in crystals and in solution. This definitely shows that at least part of the stabilization by hydroxyproline is not due to water molecules. We recently irradiated these CMP with carbon ions at the kinetic energy corresponding to irradiation of tumor cells in hadrontherapy.[100] The same ionization and fragmentation processes as for X-rays are observed, and the transferred energy to a single molecule has been estimated to be around 20 eV. We are currently extending our research to irradiation of CMP assemblies. Our last results show that proline hydroxylation stabilizes triple helix dimers (see Figure 2), presumably via H-bonds between triple helices.[103]

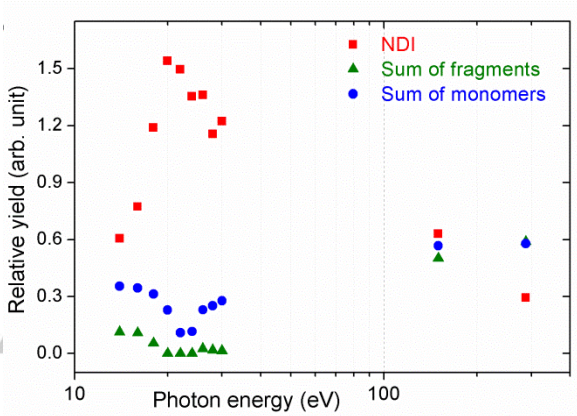

Figure 9 Relative yield of non-dissociative ionization (NDI), inter-molecular (sum of monomers) and intra-molecular (sum of fragments) fragmentation after single photoabsorption as a function of photon energy, for $[((PPG)_{10})_3+7H]^{7+}$. Reproduced from ref.[89] by permission of the PCCP Owner Societies.

4. Outlook

The relationship between structure and stability has been deeply studied for proteins, especially collagen, because of the structure-function relationship. Recent studies on collagen mimetic peptides delivered a wealth of data that elucidated the mechanisms of (de)stabilization of triple helical domains of collagen. Modelling and designing new collagen-based biomaterials is now an active area of research mainly based on the understanding of how collagen structure influences its stability.[104,105] These previous findings are also used nowadays to develop collagen-based drug delivery.[106] Open fundamental questions also remain and are to be addressed in a near future about, for instance, the role of glycine interruptions in collagen functions, the influence of the supramolecular structure on collagen stability and recognition by other molecules in the extracellular matrix, dynamics of collagen unfolding, etc. Moreover, it has been shown that interaction with non-ionizing (UV) or ionizing (VUV, X and gamma photons; electrons; ions) radiation impacts the physical and chemical properties of collagen and other peptides and proteins. When increasing the dose transferred to the molecules, the observed processes change from conformational change and cross-linking to covalent bond cleavage in the backbone and side-chains. Radical-mediated mechanisms play a big role in all these phenomena, and much detailed information has been gained by changing the nature and energy of the irradiating particle in a controlled way in gas-phase experiments. However, our understanding of the behavior of peptides and proteins under

irradiation is still limited. In particular, little is known about charge transfer and fragmentation dynamics triggered by ionization, but this gap should be filled by pump-probe experiments at the femtosecond or attosecond timescale, thanks to free electron lasers operating in the X-ray energy range.[107] Charge dynamics within amino acids[108,109] as well as DNA nucleobases and nucleosides[110] have already recently been measured. Another effect deserving future attention is the fate of protein after a localized ionization event. Designing experiments at photon energies that target a single atom is notably a direction worth exploring. Further investigation is also needed to precisely measure and potentially control the radio-induced effects on protein secondary, tertiary and quaternary structures, which would be reachable by means of ion mobility spectrometry. Indeed, this powerful technique already allows for instance identifying proteins *via* their unfolding signature,[111] but also monitoring their conformational change after collision with a rare gas or absorption of UV photons.[112]

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Keywords: collagen • proteins • structure • stability • irradiation

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